REPORT DOCUMENTATION PAGE

Form Approved OMB NO. 0704-0188

1. REPORT DATE (DD-MM-YYYY)	2. REPORT TYPE	3. DATES COVERED (From - To)
29-10-2009	Final Report	1-Sep-2006 - 31-Aug-2009

4. TITLE AND SUBTITLE
Mechanisms of the killing and development of spores of Bacillus species

5a. CONTRACT NUMBER
W911NF-06-1-0428

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER
611102

6. AUTHORS
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Research Office
P.O. Box 12211
Research Triangle Park, NC 27709-2211

10. SPONSOR/MONITOR'S ACRONYM(S)
ARO

11. SPONSOR/MONITOR'S REPORT NUMBER(S)
49324-LS.1

12. DISTRIBUTION AVAILIBILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES
The views, opinions and/or findings contained in this report are those of the author(s) and should not contrued as an official Department of the Army position, policy or decision, unless so designated by other documentation.

14. ABSTRACT
Notable findings in the final year of support include the following. 1) The proteins that comprise the mechanosensitive channels were fused to Green Fluorescent Protein (GFP) and localized in Bacillus subtilis cells. All of these proteins were in the plasma membrane, and at least two may be in a helical array. 2) The phospholipid composition of B. subtilis spores' inner membrane could be altered drastically without large effects on spore resistance or germination. 3) Spores of a cotE gerE mutant lacked most coat layers but retained a thin layer of

15. SUBJECT TERMS
Bacillus, spores, spore resistance, spore hulling, spore germination

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	15. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
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Statement of the Problem Studied and Summary of the Most Important Results

Significant progress has been made in a number of areas over the final period of this contract. These accomplishments include the following.

A) Mechanosensitive (MS) Channels. The four likely MS channel proteins we identified previously in *B. subtilis* have been fused to the *gfp* gene encoding Green Fluorescent Protein GFP. These fusions were found to largely be functional, and all four GFP fusion proteins were localized by Western blotting of cell fractions to the cell's plasma membrane, consistent with their role as MS channels. However, the proteins were not found in spores, consistent with these proteins having no role in spore maturation or germination. Interestingly, two of the MS-GFP fusions were also localized on the plasma membrane by fluorescence microscopy and these proteins appeared to be present on a helical array on the membrane, suggesting that MS channels may interact in some fashion with the cell's cytoskeleton.

B) Effects of alterations in spore inner membrane phospholipds. We obtained a large collection of isogenic *B. subtilis* strains with one or multiple deletions in enzymes for biosynthesis of various phospholipids, prepared spores from these strains and determined spore properties. Surprisingly, spores were formed relatively normally by strains that made no phosphatidylethanolamine, glycolipids, lyophosphatidylglycerol, phosphatidylserine or cardiolipin, or in some cases missing several of these lipids; in all cases the spores'inner membrane phospholipid composition was as expected based on biosynthetic genes that were absent. Loss of specific inner membrane phospholipids did have effects on spore germination and spore resistance to wet heat and oxidizing agents. However, generally these effects were $\leq$ 3-fold, with absence of lipids generally decreasing spore germination rates as well as spore resistance, while spore formation itself was only minimally affected.

C) Generation and properties of almost completely coat-less spores. *B. subtilis* strains were constructed with deletions in a gene, *cotE*, encoding a major spore coat morphogenetic protein and/or the *gerE* gene that encodes a transcription factor for many *cot* genes expressed late in sporulation. The *cotE gerE* spores in particular lacked most visible coat layers as observed by electron and atomic force microscopy, and lacked almost all readily extracted coat proteins. However, the *cotE gerE* spores did retain a thin layer of insoluble coat proteins. These largely coatless spores germinated relatively normally with nutrients, were still very resistant to wet heat, mechanical disruption and detergents at an elevated temperature, but were extremely sensitive to sodium hypochlorite.

D) Staining properties of almost coat-less spores. A major reason for the effort to prepare and characterize the *cotE gerE* spores described above was that it was hoped that these spores would be: a) generally stained less well by hydrophobic stains that often bind non-specifically to the spore's outer layer(s), presumably by adsorbing to spore coats; stains in this category include nucleic acid stains such as DAPI and acridine orange, as well as various membrane potential-sensitive dyes; and b) less autofluorescent, as this would make it easier to use GFP-reporter technology to localize low abundance proteins in dormant spores, as the spore's normally high level of autofluorescence currently makes this difficult. Indeed, both autofluorescence and non-specific surface

staining by both nucleic acid stains and membrane potential-sensitive dyes was greatly reduced in *cotE gerE* spores – probably $\geq$ 10-fold - although not eliminated.

The decreased non-specific staining of *cotE gerE* spores by membrane potential-sensitive dyes was then used to demonstrate that dormant *B. subtilis* spores have no membrane potential, although significant membrane potential is generated shortly after germination is initiated. A similar result was also obtained with wild-type spores of *B. megaterium* that non-specifically adsorb membrane potential sensitive dyes less avidly than *B. subtilis* spores.

E) X-ray scattering by B. subtilis spores. Over 40 years ago it was shown that dormant *B. subtilis* spores give a distinctive X-ray scattering pattern indicating the presence of some very ordered structure in these spores. In collaboration with Dr. Xiangyn Qiu and the NIH, we used spores of mutants lacking the DNA protective α/β-type small, acid-soluble spore proteins, as well as *cotE*, *gerE* and *cotE gerE* spores to show that one or more coat proteins under the direct or indirect control of the GerE transcription factor is responsible for the X-ray scattering by *B. subtilis* spores.

F) Physical state of water in spores. We collaborated with Dr. Bertil Halle's lab in Sweden to use NMR spectroscopy to study the physical state of water in *B. subtilis* spores. This work led to three major conclusions: 1) the water in the spore core is not in a glass-like state; 2) proteins in the spore core are rotationally immobilized; and 3) the rate of permeation of water across the spore's inner membrane is at least several orders of magnitude slower than rates of permeation across a growing cell membrane.